Supporting document 1

Risk and Technical Assessment – Application A1170

Rebaudioside MD as a steviol glycoside from *Saccharomyces cerevisiae*

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**Executive summary**

This application from Cargill Inc. seeks permission in the Australia New Zealand Food Standards Code (the Code) for a mixture of rebaudiosides M and D (Reb MD), produced from a genetically modified *Saccharomyces cerevisiae* strain.

The current permissions for steviol glycosides allow for products extracted from the *Stevia rebaudiana* Bertoni plant only. Conversely, Cargill uses a microbial fermentation production method for its Reb MD. Whilst the steviol glycosides are identical to those produced from the plant, this production method is not currently permitted in the Code.

Steviol glycosides, including rebaudiosides M and D, are already permitted for use as a food additive in the Code, with maximum permitted levels (MPL) in a variety of food categories and at GMP levels in tabletop sweeteners in Schedule 15. Cargill state that Reb MD provides improved sensory characteristics over major steviol glycosides such as rebaudioside A and stevioside, and also has similar stability, making it suitable for a wide variety of applications, functioning as a multi-purpose and low-calorie sweetener.

An acceptable daily intake (ADI) of 0-4 mg/kg bodyweight for steviol glycosides, expressed as steviol, was established by FSANZ in 2008. This ADI is appropriate for Reb MD produced from fermentation, as it is chemically the same as rebaudiosides M and D extracted traditionally from *Stevia* and would therefore follow the same metabolic pathway in humans. No new information was located subsequent to FSANZ’s previous assessments of steviol glycosides that would raise concerns regarding the safety of steviol glycosides.

Cargill’s Reb MD complies with purity specifications of JECFA. The production organism *S. cerevisiae* has a long history of food use and is not toxigenic. The production process does not give rise to any allergen concerns. Assessment of the production strain did not identify any safety concerns.

FSANZ concludes that Cargill’s Reb MD, produced from *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes, does not pose a risk to public health and safety.
# Table of contents

EXECUTIVE SUMMARY .......................................................................................................................... I

1.0 INTRODUCTION AND DESCRIPTION OF SUBSTANCE .................................................................. 2
  1.1 Objectives of the assessment ........................................................................................................... 2

2.0 FOOD TECHNOLOGY ASSESSMENT ............................................................................................. 3
  2.1 Identity and chemical properties ...................................................................................................... 3
  2.2 Information on the physical and chemical properties of Reb MD ................................................... 4
  2.3 Information on the impurity profile ................................................................................................. 5
  2.4 Information on the S. cerevisiae production organism ..................................................................... 5
  2.5 Specifics of the biosynthesis of steviol glycosides ........................................................................ 6
  2.6 Technological purpose of Reb MD ................................................................................................ 7
  2.7 Technological justification ............................................................................................................. 8
  2.8 Reb MD production process ......................................................................................................... 8
    2.8.1 Reb MD production flow chart .............................................................................................. 9
  2.9 Current permissions ....................................................................................................................... 10
  2.10 Proposed specifications for Reb MD ........................................................................................... 11
  2.11 Analytical method for detection ................................................................................................ 12
  2.12 Product stability ......................................................................................................................... 12
  2.13 Food technology conclusion ...................................................................................................... 12

3 RISK ASSESSMENT ............................................................................................................................. 13
  3.1 Safety assessment of the genetically modified production strain .................................................... 13
    3.1.1 History of use ........................................................................................................................... 13
    3.1.2 Characterisation of the genetic modification(s) ....................................................................... 14
    3.1.3 Characterisation of inserted DNA ........................................................................................... 17
    3.1.4 Genetic stability of the inserted genes ..................................................................................... 19
    3.1.5 Safety of novel proteins ......................................................................................................... 20
    3.1.6 Key findings of GM assessment ............................................................................................. 21
  3.2 Toxicological assessment of Reb MD ............................................................................................ 21
    3.2.1 Key findings of toxicological assessment ................................................................................. 22

4 REFERENCES ......................................................................................................................................... 23
1.0 Introduction and description of substance

Cargill Incorporated’s application seeks an amendment to the Australia New Zealand Food Standards Code (the Code) for approval of a purified steviol glycoside mixture for use as an intense sweetener, produced from *Saccharomyces cerevisiae* expressing steviol glycoside biosynthesis pathway genes. This purified steviol glycoside product, Reb MD, is primarily comprised of rebaudiosides M and D and may contain a mixture of the rebaudiosides A, B, C, F, stevioside, steviolbioside, rubusoside, and dulcoside A.

Schedule 3 of the Code contains specifications for steviol glycoside mixtures containing rebaudiosides M and D in S3—32, for which the Cargill Reb MD complies. S3—35 contains specifications for steviol glycosides extracted from the leaves of *Stevia rebaudiana* Bertoni using hot water extraction and enzymatic conversion. The Cargill production method is therefore not consistent with the specifications in S3—35.

Cargill’s Reb MD, similar to other already permitted steviol glycoside preparations for use in food and beverages in Australia and New Zealand, will be used as an intense sweetener for the replacement of sucrose in reduced-calorie or no-sugar-added products. Reb MD will be an alternative sweetener to parent steviol glycosides.

1.1 Objectives of the assessment

The objectives of this risk assessment were to:

- determine whether the proposed purpose is clearly stated and that Cargill’s Reb MD achieves its technological function in the quantity and form proposed to be used as a food additive
- evaluate any potential public health and safety issues that may arise from the use of Cargill’s Reb MD produced from *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes.
2.0 Food technology assessment

2.1 Identity and chemical properties

Cargill’s Reb MD is produced from a genetically modified *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes rather than the conventional method of direct extraction from the leaves of *S. rebaudiana* Bertoni. Cargill’s specification for its Reb MD is consistent with the specifications in Schedule 3 of the Code for “steviol glycoside mixture including rebaudioside M” in S3—32 if included in a steviol glycoside mixture.

Reb MD is primarily comprised of rebaudiosides M and D and may contain a mixture of the following additional glycosides in various concentrations, which are natural constituents of the *S. rebaudiana* plant: rebaudiosides A, B, C, F, stevioside, steviolbioside, rubusoside, and dulcoside A. Steviol glycosides produced by *S. cerevisiae* expressing genes for steviol glycoside biosynthesis are identical to steviol glycosides extracted from the leaves of *S. rebaudiana* and conform to the current JECFA purity criteria of ≥95% steviol glycosides with the assigned INS number. 960. The steviol glycosides produced by *S. cerevisiae* also meet the JECFA definition as being “a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose, arabinose, galactose and deoxyglucose) in any of the orientations occurring in the leaves of *S. rebaudiana* Bertoni” (JECFA 2017). Reb MD is intended be sold under the proposed trade name of ‘EverSweet’.

The distribution of steviol glycosides present in Reb MD will vary depending on the production process and final product formulation, as shown in Table 1 below.

*Table 1*  
Steviol Glycoside Composition of Reb MD

<table>
<thead>
<tr>
<th>Steviol Glycosides</th>
<th>Production batch number</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>150417-B1</td>
<td>150901-B1</td>
</tr>
<tr>
<td>Total Steviol Glycosides (%)</td>
<td>99.3</td>
<td>99.2</td>
<td>99.9</td>
</tr>
<tr>
<td>Rebaudioside D (%)</td>
<td>6.9</td>
<td>18.6</td>
<td>25.6</td>
</tr>
<tr>
<td>Rebaudioside M (%)</td>
<td>88.3</td>
<td>76.3</td>
<td>72.5</td>
</tr>
<tr>
<td>Rebaudioside A (%)</td>
<td>0.4</td>
<td>1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Rebaudioside B (%)</td>
<td>0.4</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Other glycosides</td>
<td>3.3</td>
<td>2.4</td>
<td>2.2</td>
</tr>
</tbody>
</table>

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1 In March 2018, The Codex Committee on Food Additives endorsed the listing of Steviol glycosides (INS 960)) as a food additive grouping containing Steviol glycosides from *Stevia rebaudiana* Bertoni (Steviol glycosides from Stevia) (INS 960a) and Rebaudioside A from multiple gene donors expressed in *Yarrowia lipolytica* (INS 960b(i)). The Committee also agreed to make consequential amendments to the GSFA and to the List of Codex specifications of food additives (CAC/MISC 6-2017).
2.2 Information on the Physical and Chemical Properties of Reb MD

Reb MD is a white to off-white powder with a characteristic sweet taste, consistent with the description of commercial steviol glycoside preparations published by JECFA in the most recent chemical and technical assessment (CTA) (FAO 2016). Reb MD is slightly soluble in water at room temperature. All steviol glycosides share the same backbone structure (see Figure 1) and individual glycosides differ only with respect to the type and number of sugar moieties at positions R1 and R2. Based on the structural similarities among steviol glycosides, it is expected that the physiochemical properties of Reb MD, a mixture of glycosides, will be similar to those of the individual steviol glycosides present in the final product.

![Figure 1: Backbone Structure for Steviol Glycosides](image)

Purified Reb MD meets or exceeds the ≥95% steviol glycoside purity definition for steviol glycosides from *S. rebaudiana* established by JECFA (JECFA 2017). The Chemical Abstract Service numbers (CAS), empirical formulae, molecular weights, and R1 and R2 groups for the 10 steviol glycosides that may be present in the final Reb MD product, as well as the aglycone Steviol, are summarised in Table 2.

---

2 An aglycone is the compound remaining after the glycosyl group on a glycoside is replaced by a hydrogen atom.
analyses

reaction (PCR; limit of detection of 5 ng/PCR reaction) analysis. The details of these
ng/protein band) and residual DNA

dodecyl sulphate polyacrylamide gel electrophoresis
shown to be free

Section 3
that

cerevisiae

The

requirements for cadmium and mercury in S3

the set chemical and microbiological specification parameters.

The

metal specification parameters that have been established
those defined by JECFA and the European Commission. As such, microbiological and heavy

Cargill’s Reb MD is a high purity steviol glycoside product containing no less than 95%
steviol glycosides, and all specification parameters and limits for Reb MD are consistent with
those defined by JECFA and the European Commission. As such, microbiological and heavy

2.3 Information on the Impurity Profile

Cargill’s Reb MD is a high purity steviol glycoside product containing no less than 95%
steviol glycosides, and all specification parameters and limits for Reb MD are consistent with
those defined by JECFA and the European Commission. As such, microbiological and heavy metals
specification parameters that have been established for steviol glycosides to ensure
safe use in food are applied to Reb MD. There are also additional and supplementary
requirements for cadmium and mercury in S3—4 which the Reb MD meets.

The applicant stated that Batch samples of Reb MD are routinely tested to verify compliance
with the set chemical and microbiological specification parameters.

2.4 Information on the S. cerevisiae production organism

The production organism S. cerevisiae, has a long history of safe-use in the production of
food (e.g. brewing, baking) and food ingredients (e.g. food-grade enzymes, flavourings). S.
cerevisiae (strain CD15407) (Table 3) has been engineered to express a range of enzymes
that synthesize steviol glycosides, primarily comprised of rebaudiosides M and D (see
Section 3). Analysis of the Reb MD produced from fermentation and after purification was
shown to be free of protein and nucleic acids. Protein levels were examined by sodium
dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; limit of detection of 8 to 28
ng/protein band) and residual DNA was analysed using quantitative polymerase chain
reaction (PCR; limit of detection of 5 ng/PCR reaction) analysis. The details of these
analyses from three production batches of Reb MD are provided in Section B.6.4 of the

Table 2 Additional details of Reb MD constituents

<table>
<thead>
<tr>
<th>Steviol Glycoside</th>
<th>CAS Number</th>
<th>Molecular Weight</th>
<th>Molecular Formula</th>
<th>R-Groups in Backbone Structure</th>
<th>R1</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reb A</td>
<td>58543-16-1</td>
<td>967.01</td>
<td>C₄₄H₇₆O₂₃</td>
<td>β-Glc</td>
<td>β-Glc-β-Glc(2→1)</td>
<td>β-Glc-β-Glc(3→1)</td>
</tr>
<tr>
<td>Reb B</td>
<td>58543-17-2</td>
<td>804.88</td>
<td>C₃₈H₆₇O₁₈</td>
<td>H</td>
<td>β-Glc-β-Glc(2→1)</td>
<td>β-Glc-β-Glc(3→1)</td>
</tr>
<tr>
<td>Reb C</td>
<td>63550-99-2</td>
<td>951.02</td>
<td>C₄₄H₇₆O₂₂</td>
<td>β-Glc</td>
<td>β-Glc-β-Glc(2→1)</td>
<td>β-Glc-β-Glc(3→1)</td>
</tr>
<tr>
<td>Reb D</td>
<td>63279-13-0</td>
<td>1,129.15</td>
<td>C₅₀H₆₇O₂₈</td>
<td>β-Glc-β-Glc(2→1)</td>
<td>β-Glc-β-Glc(2→1)</td>
<td>β-Glc-β-Glc(3→1)</td>
</tr>
<tr>
<td>Reb F</td>
<td>438045-89-7</td>
<td>936.99</td>
<td>C₄₃H₆₇O₂₂</td>
<td>β-Glc</td>
<td>β-Glc-β-Glc(2→1)</td>
<td>β-Glc-β-Glc(3→1)</td>
</tr>
<tr>
<td>Reb M</td>
<td>1220616-44-3</td>
<td>1,291.3</td>
<td>C₅₆H₆₇O₃₃</td>
<td>β-Glc-β-Glc(2→1)</td>
<td>β-Glc-β-Glc(2→1)</td>
<td>β-Glc-β-Glc(3→1)</td>
</tr>
<tr>
<td>Stevioside</td>
<td>57817-89-7</td>
<td>804.88</td>
<td>C₃₈H₆₇O₁₈</td>
<td>β-Glc</td>
<td>β-Glc-β-Glc(2→1)</td>
<td>β-Glc-β-Glc(3→1)</td>
</tr>
<tr>
<td>Steviolbioside</td>
<td>41093-60-1</td>
<td>642.73</td>
<td>C₃₂H₆₇O₁₃</td>
<td>H</td>
<td>β-Glc-β-Glc(2→1)</td>
<td>β-Glc-β-Glc(3→1)</td>
</tr>
<tr>
<td>Rubusoside</td>
<td>64849-39-4</td>
<td>642.73</td>
<td>C₃₂H₆₇O₁₃</td>
<td>β-Glc</td>
<td>β-Glc-β-Glc(2→1)</td>
<td>β-Glc-β-Glc(3→1)</td>
</tr>
<tr>
<td>Dulcoside A</td>
<td>64432-06-0</td>
<td>788.88</td>
<td>C₃₈H₆₇O₁₇</td>
<td>β-Glc</td>
<td>β-Glc-β-Glc(2→1)</td>
<td>β-Glc-β-Glc(3→1)</td>
</tr>
<tr>
<td>Steviol</td>
<td>471-80-7</td>
<td>318.46</td>
<td>C₂₀H₂₅O₃</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
</tbody>
</table>

CAS = Chemical Abstract Service; Glc = Glucose; Rha = Rhamnose; Xyl = Xylose.
application. These analyses also confirm that the production organism is absent from the final steviol glycoside product.

Table 3  Taxonomic Identity of Saccharomyces cerevisiae CD15407

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum</td>
<td>Ascomycota</td>
</tr>
<tr>
<td>Class</td>
<td>Saccharomycetes</td>
</tr>
<tr>
<td>Order</td>
<td>Saccharomycetales</td>
</tr>
<tr>
<td>Family</td>
<td>Saccharomycetaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Saccharomyces</td>
</tr>
<tr>
<td>Species</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>Strain</td>
<td>CD15407</td>
</tr>
</tbody>
</table>

2.5 Specifics of the biosynthesis of steviol glycosides

The biosynthesis of steviol glycosides in *S. rebaudiana* is a specific deviation from the gibberellin biosynthesis pathway found in all plants. The deviation from this pathway occurs when *ent*-kaurenoic acid is converted to steviol (Figure 2). The remaining steps of this pathway leading to the production of steviol glycosides involves the action of several different uridine diphosphate³ (UDP)-glycosyltransferases (UGTs). UGTs transfer sugar residues from activated donor molecules, such as UDP-glucose, to acceptors, such as the aglycone steviol, yielding an array of steviol glycoside molecules with differing numbers and types of sugar moieties conjugated to the steviol backbone. For this application, the pathway operational in *S. rebaudiana* has been replicated in *S. cerevisiae*, allowing the production of Reb MD via fermentation.

³ Uridine diphosphate, abbreviated UDP, is a nucleotide diphosphate. It is an ester of pyrophosphoric acid with the nucleoside uridine. UDP consists of the pyrophosphate group, the pentose sugar ribose, and the nucleobase uracil. UDP is an important factor in glycogenesis.
2.6 Technological purpose of Reb MD

Steviol glycosides extracted or derived from the leaves of *S. rebaudiana* Bertoni, including rebaudiosides M and D, are already permitted for use as food additives in the Code, with the International Numbering System (INS) assignation 960. The technological purpose of steviol glycosides as a food additive is that of an intense sweetener, which replaces the sweetness normally provided by sugars in food, without contributing significantly to their available energy. Reb MD, similar to other already permitted steviol glycoside preparations for use in food and beverages in Australia and New Zealand, will be used as a high-intensity sweetener for the replacement of sucrose in reduced-calorie or no-sugar-added products. Steviol glycosides are permitted at MPL’s in a variety of food classes and at GMP level for tabletop sweeteners in Schedule 15. The technological purpose of this particular Reb MD from Cargill does not differ from currently permitted steviol glycosides, rather it is the production method that differs.
2.7 Technological justification

The primary reason for developing alternative methods to the traditional extraction methods for steviol glycosides is that not all glycosides are produced to the same degree in the leaves of *S. rebaudiana* Bertoni. For example, stevioside is a major glycoside present in the leaves of the plant, constituting about 5 to 10% in dry leaves (JECFA, 1999), whereas rebaudiosides M and D are minor glycosides and present at much lower levels. Cargill state that Reb MD provides improved sensory characteristics over major steviol glycosides such as rebaudioside A and stevioside, and also has similar stability, making it suitable for a wide variety of applications, functioning as a multi-purpose and low-calorie sweetener.

2.8 Reb MD production process

Reb MD is a purified steviol glycoside mixture that is produced from fermentation of *S. cerevisiae* (strain CD15407) expressing steviol glycoside biosynthesis pathway genes and is manufactured in accordance with current Good Manufacturing Practices (cGMP⁴). Following fermentation, Reb MD is purified in accordance with the methodologies outlined in the CTA published by the Food and Agriculture Organization of the United Nations (FAO)/JECFA for steviol glycosides (FAO 2016). A schematic overview of the production process for Reb MD is presented in Figure 3 below. A detailed description of the manufacturing process, including the raw materials, processing aids, and equipment used in the production process can be found on page 23 of the application.

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⁴GMP limits the amount of substance that is added to food to the lowest possible level necessary to accomplish its desired effect.
2.8.1 Reb MD production flow chart

[Flowchart image]

Dextrose or sucrose
Salts
Trace Metals
Clean steam (0.2 μm filtered)

Media Sterilization
121°C, 30 min.

Filter Sterilization
Water
Vitamins and/or YE

KOH
H₂PO₄
NH₄OH
Sorage air (0.2 μm filtered)
Ant-fomn

Fermentation
28-32°C, 6-140 hrs,
ph 4.5-6.5

Heat Treatment

pH adjustment
(Optional)

Centrifugation
(Optional)

Water

Microfiltration
0.1 μm or 3.2 μm

Concentration
(Optional)

Dilution
(Optional)

Adsorption Chromatography

Evaporation

Ion-Exchange Chromatography

pH adjustment
(Optional)

Activated Carbon
(Optional)

Glycerol stock
Figure 3  Overview of the production process for Reb MD

2.9 Current permissions

Current permissions for steviol glycosides are in Schedule 3 of the Code, including S3-31; Reb M, S3-32; steviol glycoside mixtures containing Reb MD and S3-35; steviol glycosides from *Stevia rebaudiana* Bertoni. All specifications for steviol glycosides stipulate that the total steviol glycoside content must be no less than 95% on a dried basis, and refer to primary source specifications for steviol glycosides contained within section S3—2, being either S3—2(1)(b) [the FAO JECFA Monograph], S3-2(1)(c) [the Food and Chemicals Codex], or S3—2(1)(d) [European Commission Regulation No 231/2012 (EU 2012) laying down specifications for food additives]. JECFA recently issued updated tentative specifications for “Steviol glycosides from *Stevia rebaudiana* Bertoni”, which stipulate that the total percentage of steviol glycosides must be no less than 95%, where steviol glycosides are defined as “all
compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose, arabinose, galactose and deoxyglucose) in any of the orientations occurring in the leaves of *Stevia rebaudiana* Bertoni" (JECFA 2017). Cargill’s Reb MD is currently permitted for use in Mexico.

### 2.10 Proposed Specifications for Reb MD

The physical and chemical specifications for Reb MD, as presented in Table 4, were established based on those published by JECFA (JECFA 2017) and the European Commission (EU 2016) for steviol glycosides extracted from *S. rebaudiana*.

Cargill’s application contains a comprehensive product specification in section B.6 on pages 43 to 37 for their Reb MD. The product specifications are consistent with the specifications in Schedule 3 of the Code for rebaudiosides in sections S3—31, S3—32 and S3—35. They also comply with the assay and impurity specifications in the FAO JECFA Monograph 19 for “steviol glycosides from *Stevia rebaudiana* Bertoni” (JECFA 2016a). Certificates of analyses for three non-consecutive batches of Cargill’s Reb MD were provided with the application to demonstrate compliance with the defined product specifications as shown in Table 4 below.

**Table 4** Comparative rebaudioside specifications

<table>
<thead>
<tr>
<th></th>
<th>Cargill Reb MD</th>
<th>JECFA</th>
<th>the Code (S3—35)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Appearance/Description</strong></td>
<td>White/Off white powder</td>
<td>White to light yellow powder</td>
<td>White to light yellow powder</td>
</tr>
<tr>
<td><strong>Purity (%)</strong></td>
<td>≥ 95</td>
<td>≥ 95</td>
<td>≥ 95</td>
</tr>
<tr>
<td><strong>Solubility</strong></td>
<td>Soluble in water</td>
<td>Freely soluble in water</td>
<td>Freely soluble in water</td>
</tr>
<tr>
<td><strong>pH (1% solution)</strong></td>
<td>5.0-7.0</td>
<td>4.5-7.0</td>
<td>4.5-7.0</td>
</tr>
<tr>
<td><strong>Total ash (%)</strong></td>
<td>≤ 1</td>
<td>≤ 1</td>
<td>&lt;1</td>
</tr>
<tr>
<td><strong>Loss on drying</strong></td>
<td>≤ 6</td>
<td>≤ 6</td>
<td>≤ 6</td>
</tr>
<tr>
<td><strong>Residual ethanol (mg/kg)</strong></td>
<td>≤5000</td>
<td>&lt;5000</td>
<td>&lt;5000</td>
</tr>
<tr>
<td><strong>Residual methanol (mg/kg)</strong></td>
<td>≤200</td>
<td>≤200</td>
<td>≤200</td>
</tr>
<tr>
<td><strong>Lead (mg/kg)</strong></td>
<td>≤ 1</td>
<td>≤ 1.0</td>
<td>≤ 2.0</td>
</tr>
<tr>
<td><strong>Arsenic (mg/kg)</strong></td>
<td>≤ 0.2</td>
<td>≤ 1.0</td>
<td>≤ 1.0</td>
</tr>
<tr>
<td><strong>Cadmium (mg/kg)</strong></td>
<td>&lt;.001</td>
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<td>≤ 1.0</td>
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<tr>
<td><strong>Mercury (mg/kg)</strong></td>
<td>&lt;1.0</td>
<td></td>
<td>≤ 1.0</td>
</tr>
</tbody>
</table>
2.11 Analytical method for detection

The analytical methods used to confirm that Cargill’s Reb MD meets the established chemical and microbial specifications and are listed in Table B.6.3.1-1 on page 36 of the application. The methods are internationally recognised, such as the Association of Official Analytical Chemists [AOAC], U.S. Pharmacopeia [USP], and JECFA. The Reb MD content in the final product is quantified according to the JECFA HPLC method for steviol glycosides described in FAO JECFA Monograph 19 for “Steviol Glycosides from S. rebaudiana Bertoni” (JECFA, 2016a). Details of the HPLC method and chromatographic data were provided with the application. These showed that the Cargill Reb MD met and exceeded the acceptance criteria for Reb MD content, purity and moisture.

2.12 Product Stability

JECFA have concluded that “steviol glycosides, including steviol glycosides extract preparations containing higher levels of new glycosides, are thermally and hydrolytically stable for food use, including acidic beverages, under normal conditions of processing/storage” (JECFA, 2007).

To confirm that the stability of Reb MD is similar to other steviol glycosides, Cargill assessed the degradation of Reb MD compared to rebaudioside A (RA95, 95% rebaudioside A) under accelerated conditions. Samples of the final Reb MD product and rebaudioside A were analysed to determine how the degradation of the ingredients affected the taste profile.

In comparison to rebaudioside A, Reb MD showed an equivalent degradation profile with respect to steviol glycoside content when analysed using HPLC. Likewise, the sweetness degradation was similar between the products, confirming Reb MD stability to be similar to that of rebaudioside A, and other steviol glycosides.

2.13 Food technology conclusion

The food technology assessment concludes that Cargill’s Reb MD produced from S. cerevisiae (strain CD15407) expressing steviol glycoside biosynthesis pathway genes, meets the specifications currently listed in the Code. Cargill demonstrated that its commercial production of Reb MD produces a consistent product that conforms to these specifications. Its technological purpose matches that of currently permitted rebaudioside preparations produced by the currently permitted methods.
3 Risk assessment

3.1 Safety assessment of the genetically modified production strain

3.1.1 History of use

Host organism

Saccharomyces cerevisiae is a yeast, with a long history of use in food, generally associated with fermented foods and beverages (Brewer’s Yeast) and the production of leavened bakery products (Baker’s Yeast). This yeast has been classed as a Biosafety Level 1 organism, based on the United States Public Health Service Guidelines5 and has been granted Qualified Presumption of Safety6 (QPS) status by the European Food Safety Authority (EFSA). This organism is generally considered nonpathogenic to humans.

The host strain was derived from a wild type brewing yeast, that is highly related to the reference S. cerevisiae strain S288C (ATCC 204508). Prior to generating the steviol glycoside production strain, the host was modified by a series of genetic modifications to increase the efficiency of the transformation process, including the allowance of targeted integration. The taxonomy of the parental strain was then determined by DNA sequence analysis, confirming that the species was S. cerevisiae. The sequence analysed covered the 18S ribosomal RNA component of the yeast 35S pre-ribosomal RNA gene.

Gene donor organism(s)

A summary of the history of use and safety of the gene donors has been provided by the applicant. The identity of the genetic material that has been sourced from these organisms is Confidential Commercial Information (CCI) so cannot be provided in this report.

Fungal Sources

Genetic material was obtained from three yeast species: the host S. cerevisiae, Kluyveromyces lactis and Schizosaccharomyces pombe and a filamentous fungus, Ashbya gossypii. All four organisms have a history of use for the production of food or food ingredients (van Ooyen et al, 2006; Benito et al, 2015; Revuelta et al, 2016; Loira et al, 2018). They have been classified as Biosafety Level 1 organisms because they are considered non-pathogenic to humans. The yeast strains also hold QPS status with EFSA due to their history of safe use.

Plant Sources

Genes encoding some of the steviol glycoside biosynthesis enzymes were obtained from Stevia rebaudiana and Rubus suavissimus. S. rebaudiana is a member of the daisy family (Asteraceae), which includes lettuce and artichoke. Evidence suggest the leaves from the Stevia plant have been used in South America to prepare sweetened teas for more than 1500 years, thus this plant has a long history of safe use. The leaves contain steviol glycosides, which provide the sweetness to the tea. R. suavissimus is more commonly known as Chinese blackberry. A sweet tea made from the leaves of this plant is used in traditional Chinese medicine and has a long history of safe use. The leaves contain

5 https://www.cdc.gov/biosafety/publications/bmbl5/index.htm
rubusosides, which are similar to steviol glycosides.

Genes were also obtained from two common food crops *Zea mays* (corn) and *Oryza sativa* (rice). *Z. mays* has been cultivated as a food crop for ~8000 years (OECD, 2002) while *O. sativa* has been cultivated since ~15,000 BC (OECD, 1999). Both crops have a long history of safe use. Rice has been associated with food allergy in some individuals, these reactions being attributed to two specific groups of proteins (OECD, 2016). The gene used from this species does not code for any of these potentially allergenic proteins.

Genetic material was also obtained from *Arabidopsis thaliana*, commonly known as mouse cress. Although this plant is not traditionally used as food, it is ubiquitous in the environment and is not known to be pathogenic, toxigenic or allergenic to humans. The encoded proteins used from *A. thaliana* share a high degree of similarity to the same proteins found in common food crops.

*Other Sources*

A gene encoding one of the biosynthesis enzymes was obtained from a cyanobacterium from the *Synechococcus* sp. These aquatic organisms are not found in food but the enzyme is found in all species and the particular gene shares high similarity to the same enzyme in a variety of common food crops. This enzyme found in food crops is not associated with adverse effects in humans.

### 3.1.2 Characterisation of the genetic modification(s)

*Description of the introduced DNA*

The production strain for Reb MD (CD15407) was generated using standard yeast transformation techniques. Transformation was performed using linearised DNA containing only the expression cassette sequences, without any vector backbone sequences. Insertion of this DNA into the host's genome was achieved by targeted integration and homologous recombination. A total of 15 expression cassettes were transformed sequentially into the host in order to generate the final production strain. Each cassette was targeted to a specific loci in the host genome (Section 3.1.3).

The 15 unique expression cassettes were used to introduce 22 genes. Many genes were cloned into more than one expression cassette, thereby increasing the number of copies of those genes being introduced. Of the 22 genes, 7 were endogenous genes present in the host. Of the 15 genes sourced from the fungal, plants and cyanobacterium gene donors, ten of the enzymes are directly required for the biosynthesis of the steviol glycosides (Table 5). The products of the remaining genes play a role in activating or enhancing the function of the biosynthesis enzymes. Antibiotic or nutritional selective markers were present in some of the expression cassettes to allow selection of clones containing the introduced genes of interest. These markers were subsequently removed from the final production strain by Cre-lox recombination.
Table 5  Outline of the introduced genes for the biosynthesis of rebaudiosides

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGDPS</td>
<td>Geranylgeranyl pyrophosphate synthase</td>
</tr>
<tr>
<td>CDPS</td>
<td>Copalyl diphosphate synthase</td>
</tr>
<tr>
<td>KS</td>
<td>Kaurene Synthase</td>
</tr>
<tr>
<td>KO†‡</td>
<td>Kaurene Oxidase</td>
</tr>
<tr>
<td>KAH†‡</td>
<td>Kaurenoic acid hydroxylase</td>
</tr>
<tr>
<td>UGT85C2</td>
<td>UDP-Glucosyl Transferase</td>
</tr>
<tr>
<td>UGT91D2</td>
<td>UDP-Glucosyl Transferase</td>
</tr>
<tr>
<td>EUGT11</td>
<td>UDP-Glucosyl Transferase</td>
</tr>
<tr>
<td>UGT74G1</td>
<td>UDP-Glucosyl Transferase</td>
</tr>
<tr>
<td>UGT76G1</td>
<td>UDP-Glucosyl Transferase</td>
</tr>
</tbody>
</table>

† genes encoding the same enzymes from two distinct source organisms were introduced into the production strain

As discussed in Section 2.5, the steviol glycoside biosynthesis pathway is a diversion from a plants native gibberellin biosynthesis pathway. Gibberellins are isoprenoid hormones specific to plants so are not produced by yeast. However, all eukaryotes produce isoprenoids, including the intermediary compound geranylgeranyl diphosphate (GGDP). The enzymes that have been introduced into the yeast redirect the conversion of GGDP to kaurenoic acid and in turn into steviol (Figure 4). This new pathway results in the production of diterpenoid compounds, to which steviol and the rebaudiosides belong.
Figure 4  Outline of the main enzymes introduced into the yeast production strain. The blue box represents the native isoprenoid pathway in *S. cerevisiae*. The green box represents the introduced pathway that allows the yeast to produce rebaudiosides. The novel enzymes encoded by the introduced genes are indicated next to the arrows in blue text. The full names of the enzymes can be found in Table 3.1.
Two methods were used to obtain the genetic material from the gene donor organisms. Genetic material sourced from fungi was isolated by targeted amplification (PCR), as codon optimisation was not required. Genetic material sourced from non-yeast gene donors was prepared by chemical synthesis, in order to allow for codon optimisation. These changes to the genetic sequence do not result in changes to the encoded amino acid sequence. Two enzyme genes were further modified, with the aim of improving efficiency of the expressed enzymes. The changes were fully characterised and the safety of the modified proteins were considered in the safety of the novel proteins (see Section 3.1.3).

**Characterisation of the genetically modified organisms**

### 3.1.3 Characterisation of inserted DNA

Whole genome sequence (WGS) analysis was performed on the production strain using a PacBio Sequencing system. An assessment of the raw data was performed, using primer sequences to identify each loci. The summary of this assessment is presented in the order at which the DNA was transformed into the host, otherwise known as an integration event.

Locus 1: the expression cassette targeted to this locus contained seven genes associated with the production of Reb MD, each with their own promoter and terminator sequences. An antibiotic resistance gene was also included at the 5’ end of the cassette. Analysis of the targeted region of locus 1 showed that the genes were present in the same order as anticipated and the removal of the antibiotic selection gene was successful. One of the introduced genes contained several mutations and rearrangement of the sequence resulting in an early stop codon. If a truncated version of the enzyme is produced, it is unlikely that it would be functional.

Locus 2: the expression cassette targeted to this locus contained four genes associated with the production of Reb MD, each with their own promoter and terminator sequences. An antibiotic resistance gene was also included at the 5’ end of the cassette. Analysis of the targeted region of locus 2 showed that the genes were present in the same order as anticipated and the removal of the antibiotic selection gene was successful. No rearrangements or mutations were found in the integrated DNA.

Locus 3: the expression cassette targeted to this locus contained four enzyme genes associated with the production of Reb MD, each with their own promoter and terminator sequences. An antibiotic resistance gene was included at the 5’ end of the cassette. Analysis of the region around the targeted site showed there had been no integration of the enzyme genes or antibiotic resistance gene.

Locus 4: the expression cassette targeting this locus contained six genes for the production of Reb MD, each with their own promoter and terminator sequences. A selective marker gene was also included at the 5’ end of the cassette. Analysis of the targeted region showed there had been rearrangement of the introduced DNA with sequences from the locus 1 expression cassette. At one end of the locus, the integrated DNA matched the sequence from the 5’ flanking region through to the terminator of the first gene from the locus 1 expression cassette. This was then followed with genes 2-5 and the 3’ flanking sequence of the locus 4 cassette. Because of this rearrangement, there is loss of one gene from the locus 4 insert. Furthermore, the same mutated gene present in the locus 1 insert was also present in the locus 4 insert. No other mutations were observed in the rearranged locus 4 insert and the sequence analysis confirmed absence of either the antibiotic resistance gene from the locus 1 expression cassette or the selective marker gene from the locus 4 insert, in the final production strain.
Locus 5: the expression cassette targeted to this locus contained six genes associated with the production of Reb MD, each with their own promoter and terminator sequences. An antibiotic resistance gene was also included at the 5’ end of the cassette. Analysis of the region around the targeted site showed there had been no integration of the enzyme genes or antibiotic resistance gene.

Locus 6: the expression cassette targeted to this locus contained six genes associated with the production of Reb MD, each with their own promoter and terminator sequences. An antibiotic resistance gene was also included at the 5’ end of the cassette. Analysis of the region around the targeted site showed that there had been rearrangement of the introduced DNA with sequences from the locus 1 expression cassette. At one end of the locus, the integrated DNA matched the sequence from the 5’ flanking region through to the terminator of the first gene from the locus 1 expression cassette. This was then followed with genes 2-6 and the 3’ flanking sequence of the locus 6 cassette. Because of this rearrangement, there is loss of one gene from the locus 6 insert. Furthermore, the same mutated gene present in the locus 1 insert was also present in the locus 6 insert. No other mutations were observed in the rearranged locus 6 insert and the sequence analysis confirmed absence of the antibiotic resistance genes from either the locus 1 and 6 expression cassettes.

Locus 7: the expression cassette targeted to this locus contained six genes associated with the production of Reb MD, each with their own promoter and terminator sequences. No antibiotic resistance gene was present in this cassette. Analysis of the region around locus 7 showed that the genes were present in the same order as anticipated. There was no rearrangement or mutations found in integrated DNA.

Locus 8 (FAT3): the expression cassette targeted to this locus contained six enzyme genes associated with the production of Reb MD, each with their own promoter and terminator sequences. No antibiotic resistance gene was present in the cassette. Analysis of the targeted integration site identified four tandem repeats of the expression cassette had been inserted. The genes were present in the same order as anticipated. In the second repeat, there were several indels identified in three of the six genes, each resulting in a frameshift and early stop codon. The truncated proteins, if expressed, are unlikely to be functional.

Locus 9: the expression cassette targeted to this locus contained two enzyme genes associated with the production of Reb MD, each with their own promoter and terminator sequences. An antibiotic resistance gene was included at the 3’ end of the cassette. Analysis of the region around locus 9 showed there had been no integration of the enzyme genes or antibiotic resistance gene.

Locus 10: the expression cassette targeted to this locus contained two genes associated with the production of Reb MD, each with their own promoter and terminator sequences. An antibiotic resistance gene was also included at the 5’ end of the cassette. Analysis of the inserted sequence at the locus showed that the genes were present in the same order as anticipated and the removal of the antibiotic selection gene was successful. No rearrangements or mutations were found in integrated DNA.

Locus 11: the expression cassette targeted to this locus contained two genes associated with the production of Reb MD, each with their own promoter and terminator sequences. An antibiotic resistance gene was also included at the 3’ end of the cassette. Analysis of the region around the targeted site showed there had been no integration of the enzyme genes or antibiotic resistance gene.
Locus 12: the expression cassette targeted to this locus contained a single gene, flanked by a promoter and terminator. No antibiotic resistance gene was present in this cassette. Analysis of the region around the targeted site showed that the insert had been integrated as anticipated. There was no rearrangement of the inserted DNA however a mutation in the gene did result in an amino acid change (threonine to isoleucine) in the expressed enzyme. The change is not located in the active site and is unlikely to change the function of the enzyme.

Locus 13: the expression cassette targeted to this locus contained two genes associated with the production of Reb MD, each with their own promoter and terminator sequences. No antibiotic resistance gene was present in this cassette. Analysis of the region around the targeted site showed that the genes had been integrated in the same order as observed in the expression cassette. No rearrangements or mutations were found in the integrated DNA.

Locus 14: the expression cassette targeted to this locus contained two enzyme genes associated with the production of Reb MD, each with their own promoter and terminator sequences. An antibiotic resistance gene was included at the 3’ end of the cassette. Analysis of the region around the targeted site showed there had been no integration of the enzyme genes or antibiotic resistance gene.

Locus 15: the expression cassette targeted to this locus contained three genes associated with the production of Reb MD, each with their own promoter and terminator sequences. No antibiotic resistance gene was present in this cassette Analysis of the region around the targeted site showed there had been no integration of the enzyme genes.

Results from the sequencing have shown that of the 22 genes that were targeted for introduction, 17 were successfully integrated. Of the enzyme genes that were not integrated, endogenous genes already exist in the host and would be able to be utilised for the generation of the product. This is further substantiated in the evidence provided by the applicant showing that Reb MD is being produced. Although mutations were identified in some genes, resulting in frameshifts and potential expression of truncated and non-functional proteins, most of these genes were introduced in multiple expression cassettes with at least one copy of a fully functional gene inserted for each of the 17 successful gene inserts. The data also confirmed there were no antibiotic resistance genes present in the final production strain.

3.1.4 Genetic stability of the inserted genes

The methodology used to introduce the genetic material into the host involves homologous recombination followed by selection of transformants using antibiotic or nutritional selective pressure. This ensures that the DNA is integrated. The selective markers are then removed. To ensure that the production strain remains stable without selective pressure, the cells are monitored for stability over a 3-stage seed train before a production run is performed. This involves a phenotypic analysis, measuring the production rate of Reb MD, after an initial inoculum culture is transferred to two successive higher volume cultures, during the scale-up prior to a production run.

Data were provided from several phenotypic analyses comparing Reb MD levels from both a commercial production site and a lab-based fermentation run that covered 21-22 generations. The level of Reb MD produced over the generations was consistent across sites and across five independent fermentation runs. Although this data does not directly demonstrate the genetic stability of the introduced genes, the data does show that the introduced genes are being consistently expressed and encoding functional proteins over the
generations analysed.

3.1.5 Safety of novel proteins

In the consideration of whether the novel proteins being evaluated in this application are safe, it is important to take into account that the expressed proteins, along with the genetically modified yeast, are removed during the purification of the rebaudiosides. Due to the degree of purification of the final food, it is highly unlikely that novel protein or DNA will be present (see Section 2.4).

It is also important to consider that a large and diverse range of proteins are ingested as part of the normal human diet, which do not cause adverse effects. Only a small number of dietary proteins have the potential to cause adverse health effects, either because they have anti-nutrient properties or they can trigger allergic reactions in some individuals (Delaney et al. 2008). Furthermore, proteins perform a wide range of functions in humans. To encompass the range of type and function, the safety assessment of any novel proteins must consider if there is a history of safe use, whether there are any potential toxic, anti-nutrient or allergenic effects and whether the protein is susceptible to digestion.

History of safe use

The genes introduced into the S. cerevisiae host encode a range of fungal, plant and cyanobacterial proteins. The expressed proteins have been sourced from organisms commonly used as or in food or have high sequence similarity to proteins commonly found in food.

Bioinformatic analysis for potential allergenicity

The applicant provided results of an in silico analysis comparing the introduced proteins to known allergenic proteins in the Food Allergy Research and Resource Program dataset, which is available through AllergenOnline7 (University of Nebraska). The database at the time of the search contained 2035 sequences (v.17).

Three types of comparisons were done:

a) Full length sequence search – a FASTA alignment was performed comparing the whole sequence to the database entries. Significant homology was determined when there was more than 50% similarity between the query protein and database entry, with the E-value threshold set at 1.

b) 80-mer sliding window search – a FASTA alignment was performed comparing all contiguous 80 amino acids within the novel amino acid sequences to the database entries (Pearson and Lipman 1988). Matches were identified if there was greater than 35% homology.

c) 8-mer exact match search – A FASTA alignment was performed comparing contiguous 8 amino acids within the novel amino acid sequences to the database entries (FAO/WHO 2001). Matches were identified if there was 100% homology.

Results from the allergen search did not identify any biological significant similarity of the novel proteins to the known allergens contained within the database.

7 http://www.allergenonline.org/
Bioinformatic analysis for potential toxicity

The applicant provided results from in silico analyses comparing the introduced proteins to known protein toxins identified in two custom UniProt databases: animal venom proteins and toxins\(^8\) and virulence factors\(^9\). A BLASTP algorithm using the default BLOSUM62 scoring matrix was used and matches were identified if homology was ≥ 35%. The results showed there was no biological significant homology between the novel proteins expressed in the production strain to known toxins or virulence factors.

3.1.6 Key findings of GM assessment

CD15407 was generated by transformation of 22 genes contained on 15 expression cassettes. Molecular analysis has shown that 17 of the 22 genes were successfully integrated and some of these genes are present in more than one insert site. Analysis of Reb MD expression suggests that the genes that have been integrated are stable and are inherited from generation to generation. Bioinformatic analysis has confirmed that the proteins expressed in CD15407 show no biologically significant similarity to known toxins or allergens.

3.2 Toxicological assessment of Reb MD

The safety of steviol glycosides has been assessed previously by FSANZ. FSANZ first assessed steviol glycosides in 2008 (Application A0540) and established an ADI of 0-4 mg/kg bw/day steviol, based on findings in a two-year study in rats. At that time, only ten steviol glycosides were known; stevioside, dulcoside, steviolbioside, rubudioside, and rebaudiosides A, B, C, D, E and F. The ADI is expressed as steviol because all known steviol glycosides share a common metabolic pathway, and are hydrolysed to steviol at similar rates.

The FSANZ ADI is consistent with the ADI established by JECFA at the 69\(^{th}\) meeting held in the same year, and published in 2009. JECFA re-assessed steviol glycosides at the 82\(^{nd}\) meeting in 2016 and confirmed the existing ADI.

FSANZ updated the hazard assessment of steviol glycosides in 2011 (Application A1037) but did not find reason to change the ADI established in 2008. Reb M was assessed and approved in 2015 (Application A1108). FSANZ expanded the definition of steviol glycosides to include all steviol glycosides found in the leaves of \textit{S. rebaudiana} Bertoni in February 2017 (Application A1132). Most recently, the safety of Reb M was re-assessed in Application A1157 (October 2018). A number of new studies have been assessed in the course of these Applications, but no evidence has been found to justify a change to the ADI of 0-4 mg/kg bw/day steviol set in 2008.

Two unpublished studies conducted to determine whether the steviol glycosides that are the subject of this Application are metabolised in the same way as steviol glycosides extracted from the leaves of \textit{S. rebaudiana} Bertoni are summarised below.

\textit{In vitro fermentation metabolism of steviol glycosides and steviol glycoside isomers under anaerobic conditions.} (Bonnema and Gaspard 2014) Regulatory status: Non-GLP.

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\(^8\) https://www.uniprot.org/uniprot/?query=taxonomy%3A%22Metazoa%22+AND+%5b33208%5d%22+AND+%28keyword%3A%22toxin%22+OR+annotation%3A%22type%22%3E%22issue%22+AND+venom%22%29+AND+reviewed%3Ay

\(^9\) https://www.uniprot.org/uniprot/?query=keyword:KW-0843
The test articles for this study were a rebaudioside A standard extracted from *S. rebaudiana*, and the mother liquor from the final crystallization step in the production of two separate lots of Reb MD. This mother liquor contains relatively enriched concentrations of steviol glycoside isomers that have not previously been tested for degradation, because they are present at very low concentrations in extracts made directly from *S. rebaudiana*. The test system comprised homogenized fecal samples from healthy donors (2 male and 2 female). Assays were conducted in triplicate. Blank assays, containing no steviol glycosides, were also conducted as negative controls. Experimenter were blinded to the identity of the test articles which were provided as dry powders and hydrated prior to fermentation. Fecal homogenate was prepared in phosphate-buffered saline. Fermentation was conducted under anaerobic conditions in peptone fermentation media. The test articles were added at 100 mg/bottle and fecal inoculum that had been treated with reducing solution was added at 10 mL/bottle. Bottles were incubated in a circulating water bath at 37°C. At 0, 4, 8 and 24 h, gas production and pH were measured and a sample of the bottle contents was collected. Fermentation in the samples was stopped by addition of an equal volume of methanol containing 5% ammonium hydroxide with vortexing and sonication. Samples were then centrifuged and the supernatant frozen at -80°C until analysis of steviol glycosides and steviol by UHPLC/UV. All steviol glycosides were degraded to steviol by the 20 h timepoint. It was concluded that all of the steviol glycosides present in Reb MD are degraded to steviol in a manner similar to that of Reb A.

In vitro fermentation metabolism of steviol glycosides and isomers produced from fermentation. (Bonnema et al. 2017) Regulatory status: Non-GLP.

The metabolism and analytical methods used in this study were essentially as those used in the previous study, with an additional analytical timepoint at 48 h. Assays were conducted on a blank, Reb A as the positive control, mother liquor containing 37.3% Reb D and 59.5% Reb M, mother liquor containing 8.4% Reb D and 88.4% Reb M, a leaf-based mix of 37.3% Reb D and 59.5% Reb M, and a leaf-based mix containing 8.4% Reb D and 88.4% Reb M. Results showed that degradation of the steviol glycoside isomers in the mother liquor was very similar to degradation of Reb A and also similar to the leaf-based mixes of Reb D and Reb M. It was concluded that regardless of the origin of the steviol glycoside, metabolism by large intestinal bacteria is the same.

3.2.1 Key findings of toxicological assessment

Steviol glycosides have previously been assessed and approved by FSANZ and no new evidence was submitted, or located from other sources, that would justify a change to the ADI of 0-4 mg/kg bw/day steviol set in 2008.

The applicant provided *in vitro* studies which show that steviol glycosides in Reb MD undergo similar large intestinal bacterial degradation to steviol as Rebaudiosides A, M and D derived from *S. rebaudiana*.
4 References


Accessed 6 Nov 2018


